Example 3: <u>Preparation of Oligonucleotide-Modified Gold Nanoparticles</u>

Gold colloids (13 nm diameter) were prepared as described in Example 1. Thiol-oligonucleotides [$HS(CH_2)_6OP(O)(O^-)$-oligonucleotide] were also prepared as described in Example 1.

The method of attaching thiol-oligonucleotides to gold nanoparticles described in Example 1 was found not to produce satisfactory results in some cases. In particular, when long oligonucleotides were used, the oligonucleotide-colloid conjugates were not stable in the presence of a large excess of high molecular weight salmon sperm DNA used as model for the background DNA that would normally be present in a diagnostic system. Longer exposure of the colloids to the thiol-oligonucleotides produced oligonucleotide-colloid conjugates that were stable to salmon sperm DNA, but the resulting conjugates failed to hybridize satisfactorily. Further experimentation led to the following procedure for attaching thiol-oligonucleotides of any length to gold colloids so that the conjugates are stable to high molecular weight DNA and hybridize satisfactorily.

A 1 mL solution of the gold colloids (17nM) in water was mixed with excess (3.68 µM) thiol-oligonucleotide (28 bases in length) in water, and the mixture was allowed to stand for 12-24 hours at room temperature. Then, 100 µL of a 0.1 M sodium hydrogen phosphate buffer, pH 7.0, and 100 µL of 1.0 M NaCl were premixed and added. After 10 minutes, 10 µL of 1% aqueous NaN_3 were added, and the mixture was allowed to stand for an additional 40 hours. This "aging" step was designed to increase the surface coverage by the thiol-oligonucleotides and to displace oligonucleotide bases from the gold surface. Somewhat cleaner, better defined red spots in subsequent assays were obtained if the solution was frozen in a dry-ice bath after the 40-hour incubation and then thawed at room temperature. Either way, the solution was next centrifuged at 14,000 rpm in an Eppendorf Centrifuge 5414 for about 15 minutes to give a very pale pink supernatant containing most of the oligonucleotide (as indicated by the absorbance at 260 nm) along with 7-10% of the colloidal gold (as indicated by the absorbance at 520 nm), and a compact, dark, gelatinous residue at the bottom of the tube. The supernatant was removed, and the residue was

resuspended in about 200 µL of buffer (10 mM phosphate, 0.1 M NaCl) and recentrifuged. After removal of the supernatant solution, the residue was taken up in 1.0 mL of buffer (10 mM phosphate, 0.1 M NaCl) and 10 µL of a 1% aqueous solution of NaN_3. Dissolution was assisted by drawing the solution into, and expelling it from, a pipette several times. The resulting red master solution was stable (*i.e.*, remained red and did not aggregate) on standing for months at room temperature, on spotting on silica thin-layer chromatography (TLC) plates (see Example 4), and on addition to 2 M NaCl, 10 mM $MgCl_2$, or solutions containing high concentrations of salmon sperm DNA.

Example 4: <u>Acceleration Of Hybridization of Nanoparticle-Oligonucleotide Conjugates</u>

The oligonucleotide-gold colloid conjugates I and II illustrated in Figure 11 were prepared as described in Example 3. The hybridization of these two conjugates was extremely slow. In particular, mixing samples of conjugates I and II in aqueous 0.1 M NaCl or in 10 mM $MgCl_2$ plus 0.1 M NaCl and allowing the mixture to stand at room temperature for a day produced little or no color change.

Two ways were found to improve hybridization. First, faster results were obtained by freezing the mixture of conjugates I and II (each 15 nM contained in a solution of 0.1 M NaCl) in a dry ice-isopropyl alcohol bath for 5 minutes and then thawing the mixture at room temperature. The thawed solution exhibited a bluish color. When 1 µL of the solution was spotted on a standard C-18 TLC silica plate (Alltech Associates), a strong blue color was seen immediately. The hybridization and consequent color change caused by the freeze-thawing procedure were reversible. On heating the hybridized solution to 80°C, the solution turned red and produced a pink spot on a TLC plate. Subsequent freezing and thawing returned the system to the (blue) hybridized state (both solution and spot on a C-18 TLC plate). In a similar experiment in which the solution was not refrozen, the spot obtained on the C-18 TLC plate was pink.

A second way to obtain faster results is to warm the conjugates and target. For instance, in another experiment, oligonucleotide-gold colloid conjugates and an

oligonucleotide target sequence in a 0.1 M NaCl solution were warmed rapidly to 65°C and allowed to cool to room temperature over a period of 20 minutes. On spotting on a C-18 silica plate and drying, a blue spot indicative of hybridization was obtained. In contrast, incubation of the conjugates and target at room temperature for an hour in 0.1 M NaCl solution did not produce a blue color indicative of hybridization. Hybridization is more rapid in 0.3 M NaCl.

Example 5: <u>Assays Using Nanoparticle-Oligonucleotide Conjugates</u>

The oligonucleotide-gold colloid conjugates 1 and 2 illustrated in Figures 12A-F were prepared as described in Example 3, and the oligonucleotide target 3 illustrated in Figure 12A was prepared as described in Example 2. Mismatched and deletion targets 4, 5, 6, and 7 were purchased from the Northwestern University Biotechnology Facility, Chicago, IL. These oligonucleotides were synthesized on a 40 nmol scale and purified on an reverse phase C18 cartridge (OPC). Their purity was determined by performing ion exchange HPLC.

Selective hybridization was achieved by heating rapidly and then cooling rapidly to the stringent temperature. For example, hybridization was carried out in 100 µL of 0.1 M NaCl plus 5 mM $MgCl_2$ containing 15 nM of each oligonucleotide-colloid conjugate 1 and 2, and 3 nanomoles of target oligonucleotide 3, 4, 5, 6, or 7, heating to 74°C, cooling to the temperatures indicated in Table 1 below, and incubating the mixture at this temperature for 10 minutes. A 3 µL sample of each reaction mixture was then spotted on a C-18 TLC silica plate. On drying (5 minutes), a strong blue color appeared if hybridization had taken place.

The results are presented in Table 1 below. Pink spots signify a negative test (*i.e.*, that the nanoparticles were not brought together by hybridization), and blue spots signify a positive test (*i.e.*, that the nanoparticles were brought into proximity due to hybridization involving both of the oligonucleotide-colloid conjugates).